Chapter IV

*In vitro and in vivo* evaluation of the health promoting attributes of *Enterococcus faecium* MBTU-PIF1

4.1 Introduction

Human beings, like all animals, play host to many types and high numbers of microbes on our skin, in our mouth, in women’s vaginal tracts, and all the way through our gastrointestinal tract. These microorganisms that colonize our bodies is that they interact dynamically with each other and with our human cells. The gastrointestinal tract serves to bridge the gap between “inside the body” and “outside the body”. Along this interface, microbes and foreign antigens colonizing or passing through the GI tract interact with important components of the immune system. This interaction serves to prime or stimulate the immune system for optimal functioning. Many functions are associated with the bacterial GI tract communities and their interaction with the host system including roles in host nutrition, intestinal epithelial development and activity, education of the immune system, maintenance of the integrity of the mucosal barrier, and contribution to drug and xenobiotic metabolism. Molecular approaches revealed a relatively stable composition of gastrointestinal microbiota in individual adults, but they appeared to vary considerably when different individuals were compared (Zoetendal et al., 1998). Moreover, host development, host genotype, and environmental factors influence the composition of the microbiota, illustrating how microbiologically challenging this environmental niche is (Zoetendal et al., 2004). The definition of probiotics has been evolving with the increasing understanding of the mechanisms by which they influence human health. De Vuyst et al. (2004) described a probiotic as a preparation of or a product containing viable, defined microorganisms in
sufficient numbers, which alter the microbiota in a compartment of the host that exert beneficial effects in the host. This definition incorporates the beneficial effects of probiotic microorganisms. Probiotic consumption exert a myriad of beneficial effects, such as enhanced immune response, balancing of colonic microbiota, vaccine adjuvant effects, reduction of fecal enzymes implicated in cancer initiation, treatment of diarrhoea associated with travel and antibiotic therapy, control of Rotavirus and Clostridium difficile induced colitis, and prevention of ulcers related to Helicobacter pylori. Probiotics are also implicated in the reduction of serum cholesterol, the antagonism against food-borne pathogens and tooth decay organisms, the amelioration of lactose malabsorption symptoms as well as candidiasis and urinary tract infections (Saavedra, 2011).

Probiotic supplements can directly or indirectly influence the populations or activities of our colonizing microbes and thereby can impact human health. It is important to note that these probiotic cultures are to exert their effects on host physiology within a highly complex ecosystem that is colonized by a myriad of endogenous microbes. Probiotics favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection.

The physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance (Helland et al., 2004; Parvez et al., 2006).

Probiotic strains assimilate cholesterol for their own metabolism. The organisms bind to the cholesterol molecule, degrading it to its catabolic
products. Thus the cholesterol level gets reduced indirectly by deconjugating the cholesterol to bile acids and reducing the body pool.

The inability of adults to digest lactose, or milk sugar, is prevalent worldwide. Consumption of lactose by those lacking adequate levels of lactase produced in the small intestine can result in symptoms of diarrhoea, bloating, abdominal pain and flatulence. These symptoms are due to undigested lactose reaching the large intestine and being fermented by the colonic microbes. These microbes can produce gases and products that lead to watery stool. The inability to consume dairy products can potentially compromise calcium intake, threatening bone health. Yoghurt was found to aid digestion of lactose because the Lactic Acid Bacteria used to make yoghurt deliver lactase to the small intestine, where it breaks down the lactose before it reaches the colon. In addition to yoghurt starter bacteria, *L. acidophilus* and Bifidobacteria have been shown by several studies to improve digestion of lactose, although generally to a lesser extent than the yoghurt starter cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.

Probiotic bacteria are shown to promote the endogenous host defense mechanisms. Probiotics stabilize the gut microflora and promote nonimmunologic gut defence. They improve the intestine's immunologic barrier by enhancing humoral immune responses. Moreover, probiotic bacteria have been shown to stimulate nonspecific host resistance to microbial pathogens and modulate the host's immune responses to potentially harmful antigens with a potential to down-regulate hypersensitivity reactions (Isolauri *et al.*, 2001). Probiotics in healthy hosts can reconstitute the intestinal mucosa through reduction of its permeability and can strengthen the local immune response, particularly through the IgA and systemic immunity by acting as an adjuvant (Galdeano, 2011). IgA is an important
antibody for prevention from foreign antigen on mucosal sites providing an immunological barrier against foreign matter by preventing the absorption of such material into the mucosal epithelium. IgM and IgG are the important antibodies for prevention from pathogenic microorganisms in serum. Determination of IgA, IgM and IgG can be used as the markers of humoral immunomodulatory function. IL-12 activates Th1 cells leading to the activation of cellular immunity and IFN gamma and IL-2 are markers of cellular immunomodulatory function preventing infection and allergy (Kimura et al., 2006). It has been proved in many studies that probiotic bacteria have the potential to prevent the onset of allergic diseases. Possible mechanisms include, enhancement of gut barrier function, restoration of intestinal permeability, production of interleukins and cytokines that are involved in regulation of IgE production. Probiotic modulation of host immunity is a very promising area of research.

According to guidelines for evaluation of probiotics for food, appropriate in vitro and animal assessments must be conducted to better understand the physiological attributes of the strain. When selecting probiotics for potential use, it is important to consider that not all bacteria with probiotic potential behave the same. Different species of the same bacterial genus may act similarly in laboratory conditions; however, their in vivo effects may be dissimilar (Lefebvre S, BARK).

Several mechanisms of action have been proposed to explain how probiotic organisms may act within a host to yield beneficial effects. These can be grouped into two categories: suppression of certain gastrointestinal (GI) microflora, and immunomodulation of the host. Possible mechanisms of floral suppression include release of antimicrobial compounds, competition for nutrients, or competition for adhesion sites in the intestine. Suggested
mechanisms of immunomodulation include an increase in antibody or cytokine production, modulation of phagocytosis and stimulation of nonspecific immunity. Animal and some human studies have shown an effect of yoghurt or Lactic Acid Bacteria on enhancing levels of certain immunoreactive cells (e.g. macrophages, lymphocytes) or on regulation of immune factors (cytokines, immunoglobulins, interferon).

4.2 Review of Literature

4.2.1 The mucosal barrier, microflora of gastrointestinal tract and significance to host health

Mucosal membrane surfaces provide the strategic interface between the internal and external world and contain a large and variable antigenic load (Didierlaurent et al., 2002). Most infectious diseases are acquired by, or affect, mucosal surfaces such that preventive and protective host defenses require mucosal response. Mucous surfaces are protected by many defence mechanisms that ensure a permanent and effective protection. They include the production of secretory IgA, the production of mucus, cytoprotective peptides, defencins etc. Mucous membranes of the body are in direct contact with the outside environment and they are colonized by a large number of different bacteria. Through mucous membranes, the organism is in permanent contact with different antigens. Protection against potentially harmful agents is ensured by many factors, including saliva, gastric acid, peristalsis, mucus, intestinal proteolysis, intestinal flora and epithelial cell membranes with inter cellular junctional complexes (Sanderson et al., 1993). Maturation of gut defense barrier includes appearance of mucosal proteins and the development of the intestinal flora (Isolauri et al., 2001). The intestinal mucous layer is considered to be one of the biggest immune organs of the body where all types of immunocompetent cells have been
identified (Brandzaeg et al., 1999). The surface of mucosal membrane is protected by a local adaptive immune system. The gut associated lymphoid tissue represents the largest mass of lymphoid tissue in the human body and constitutes an important element of the total immunologic capacity of the host (Isolauri et al., 2001). The gut immune system contains the highest numbers of macrophages, plasma cells, and T lymphocytes in the body. Mucosal immune response is characteristically directed towards a T helper type 2 (Th2) cytokine pattern dominated by production of interleukin 4 (IL-4) and IL-5, which supports B-cell differentiation and the development of antibodies. Response to infection is mediated by secretory IgA antibodies and mucosal cytotoxic T cells and a highly developed innate immune response (Rakoff-Nahoum et al., 2004).

The secretory immune system protects the mucous epithelial surface by immune exclusion where specific secretory IgA antibodies are produced against luminal antigens to prevent later response (Herich and Levkut, 2002). Lymphocytes in the B-cell lineage can induce intestinal epithelial enterocytes into M cell like epithelial cells which can transfer particles and microbes from the gut lumen to the underlying follicles (Isolauri et al., 2001).

Indigenous microflora markedly affects the structure of the host mucous, its functions, and development of the whole immune system (Herich and Levkut, 2002). The gut microflora elicit specific immune response at a local and systemic level (Isolauri et al., 1993; Kalia et al., 1992) The intestinal flora acts as an important antigenic stimulus for the maturation of gut associated lymphoid tissue and participate in tolerance induction (Helgeland et al., 1996; Shroff et al., 1995). Gut microbial population can synthesize a wide range of lipid molecules that vary in chemical structure from short chain fatty acids such as butyrate, acetate and propionate to
polyunsaturated fatty acids (conjugated lenolenic acid and isomers) involved in regulating apoptosis and immune response (Bassaganya-Reira, 2012). The presence of large populations of commensal bacteria has a major influence on the development of immune response that is only now beginning to be studied in detail. Commensal flora directly activates the mucosal immune system leading to the development of Peyer’s patches and to IgA plasma cells and CD4+T cells in the lamina propria. Experimental studies have shown that mucosal immune response is primed in the neonate and that responses generated at this time are retained in later life. Generally, microflora influence maintenance of intestinal homeostasis through direct effects on the development of organized lymphoid tissue (Rhee et al., 2004) and prevention of inflammation.

4.2.2 Health benefits of Probiotics

The idea of using microbes to promote a good health and to prevent disease is not new. For centuries, microbial probiotics have been used, without specific knowledge of how they function, as supplementary diets to promote the good health. The study regarding beneficial effect of probiotics was carried out by Metchnikoff in the early 1900’s. He pointed out that some small living organisms or substances produced in fermented food products might influence the balance of intestinal microflora (Chukeatirote, 2003). Probiotics have been currently used to improve the health of humans and animals in various aspects. Brief review of the health benefits of probiotics are given below.

4.2.2.1 Improvement of intestinal microflora balance.

Use of probiotics is likely to be the most natural and safe means for maintaining the optimum gut flora balance between the friendly bacteria and
pathogenic bacteria. The friendly bacteria will prevent colonization of bacterial pathogens by competing for essential nutrients or attachment sites (Chukeatirote, 2003). It has been reported that the manipulation of gut microflora with probiotic bacteria can regulate gut homeostasis and barrier function partly by production of bacterial metabolites (Bassanganya-Reira et al., 2012). According to Herich and Levkut, (2002) Lactic Acid Bacteria ensure the balance in the composition of the gut microflora, and through their activity are able to maintain the integrity of the gut mucous membrane.

4.2.2.2 Role of probiotics in lowering of serum cholesterol level

Resistance to bile salts is generally considered as an essential property for probiotic strains to survive the conditions in the small intestine. Bile salts are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form. Bile functions as a biological detergent that emulsifies and solubilizes lipids and play important role in fat digestion. This detergent property of bile also confers potent antibacterial activity, through the dissolution of bacterial membranes (Begley et al., 2005). Conjugated and unconjugated bile acids are absorbed by passive diffusion along the entire gut and by active transport in the terminal ileum. The reabsorbed bile acids are resecreted into bile. Bortolini et al. (1997) reported that approximately 5% of the total bile acid pool gets extensively modified by the indigenous intestinal bacteria. The transformation includes deconjugation which is catalyzed by bile salt hydrolase enzymes and the resulting bile acids are in the unconjugated or deconjugated form (Batta et al., 1990; Begley et al., 2006; Sudha et al., 2009). According to De Smet et al. (1995), bile salt hydrolytic (BSH) activity may contribute to resistance of Lactobacilli to the toxicity of conjugated bile salts in the duodenum and therefore is an important
colonization factor. It has also been suggested that BSH enzyme might be a shock protein that enables Lactobacilli to survive the intestinal bile stress. Bile salt deconjugation is considered one mechanism involved in the reduction of serum cholesterol level.

Hypercholesterolemia occurs when there is an elevated level of total cholesterol in the bloodstream. It is the result of high levels of low density lipoprotein (LDL) as compared to high density lipoprotein (HDL). LDL leaves behind fatty deposits in the blood vessels, accumulation of which can congests blood vessels and blocks blood supply to the organs. Hypercholesterolemia hardens and narrows blood vessels in various parts of the body leading to chest pain, heart attack and stroke. With the emergence of a more health conscious society, the role of probiotic food products has gained attention (Perdigon et al., 1991). According to Taranto et al. (2003) probiotic Lactic Acid Bacteria might be a more natural way to decrease cholesterol in humans. Every reduction in serum cholesterol concentration is associated with an estimated 2% to 3% reduction in risk for coronary heart disease. Regular intake of fermented milk containing an appropriate strain of *Lactobacillus acidophilus* has the potential of reducing risk for coronary heart disease by 6 to 10% (Sudha et al., 2009). High cost of medicines is a limitation for pharmacological therapy in treating hypercholesterolemia and related cardiovascular diseases. Dietary therapy as alternative strategy for treating hypercholesterolemia has received considerable attention of scientific community.

Many LAB have the ability to reduce cholesterol level in the growth medium containing bile salts. The ability to reduce cholesterol may be influenced by numerous factors, such as the kind of medium, presence of bile salts, the viability and number of bacterial cells. Rasic et al. (1992) observed
the ability of three *Lactobacillus acidophilus* strains to uptake cholesterol in MRS during 18h culture in 37°C. According to the study conducted by Lin and Chen, (2000), six strains of *Lactobacillus acidophilus* were able to remove 20% to 57% cholesterol in the presence of oxgall, 43% to 71% in the presence of cholic acid and 11% to 52% in the presence of taurocholic acid. The various mechanisms proposed for the purported cholesterol lowering action of probiotics include physiological actions of the end products of short chain fatty acid fermentation, cholesterol assimilation by the bacteria, cholesterol binding to the bacterial cell wall, and enzymatic deconjugation of bile acids (Pereira and Gibson, 2002). Walker and Gilliland (1993) and Brashears *et al.*, (1998) reported that the uptake of cholesterol by LAB resulted from its precipitation together with deconjugated bile salts and not from its removal by bacterial cells.

Anderson and Gilliland, (1999) found that significant reduction in serum cholesterol on examining the effect of consumption of one daily serving of yoghurt on serum lipids by performing controlled clinical studies. Liong and Shah, (2005) screened eleven strains of Lactobacilli and analyzed bile salt deconjugation ability, bile salt hydrolase activity and co-precipitation of cholesterol with deconjugated bile. It is reported that cholesterol removal from culture media was a result of precipitation of cholesterol with free bile acids, formed in the media because of the activity of the bacterial enzyme bile salt hydrolase (Klaver and Van der Meer, 1993; Parvez *et al.*, 2006).

### 4.2.2.3. Alleviation of symptoms of lactose intolerance

Lactose intolerance is a physiological state in human beings where they lack the ability to produce an enzyme named lactase or β galactosidase.
This enzyme is essential to assimilate the disaccharide in milk that needs to be split into glucose and galactose (Wadher et al., 2010). Inability to digest lactose in milk poses a problem in newborn infants and people with lactose intolerance express abdominal discomfort, diarrhoea, cramps, flatulence, nausea, vomiting etc. β galactosidase enzyme is produced by most Lactobacilli and is widely used in dairy industry. This enzyme hydrolyzes lactose, the main carbohydrate in milk. The bacterial species currently used by the dairy industry which produces β galactosidase enzyme belong to the genera of Lactobacillus and Bifidobacterium and comprise a very limited collection of strains (Fernandez et al., 1999).

4.2.2.4 Effect of probiotics on the haematological and biochemical parameters

Lactic Acid Bacteria can transiently colonize the intestine of animal and human and exert beneficial effects. Bacterial strains have different biological activities in the host depending on the biochemical composition on the surface of microorganisms. The effect of probiotic supplementation on the health of animals and humans have been reported in many studies. De Smet et al. (1994) suggested that probiotic bacteria increase the synthesis of unconjugated bile acids, thus contributing to the regulation of serum cholesterol, which is a precursor of bile acids. The result of increased synthesis of these acids reduces the concentration of cholesterol. In a well controlled clinical trial for 8 weeks in overweight subjects, daily consumption of 450 ml fermented yoghurt with Streptococcus thermophilus and Enterococcus faecium resulted in an 8.4% reduction in LDL and an increase in fibrinogen levels (Agerholm-Larsen et al., 2000).

Adding probiotics to the diet lowered the cholesterol concentrations, in the liver and blood serum in rats significantly. There are indications that
probiotic supplementation of the diet lead to changes in serum lipid profiles. A single dose of *Lactobacillus* administered intragastrically improved weight gain and food conversion efficiency in birds (Khan *et al.*, 2007; Liu *et al.*, 2007; Zulkifii *et al.*, 2000). According to Salma *et al.* (2007) application of *Rhodobacter capsulatus* into diet can reduce cholesterol concentration and improve the ratio of unsaturated fatty acids to saturated fatty acids in broiler meat. Kunavue and Lien, (2012) studied the positive effects of probiotic on the growth performance and blood parameters in pigs. The effects of *Enterococcus faecium* M 74 on parameters such as lipid profile, serum calcium and inorganic phosphorus, as well as haematological parameters were examined in hens by Capcarova *et al.*, (2010). Kil *et al.* (2004) studied the effect of continuous feeding of probiotic in pigs and found that the digestibility of protein, fat and calcium in probiotic fed pigs were greater than in control group. Panda *et al.* (2006) confirmed that addition of the probiotic strain *Lactobacillus sporogens* had a positive effect on bone breaking strength and bone ash content and could be attributed to the favourable environment in intestinal tract created by the feeding of probiotic strain. Probiotic supplementation can help to digest and absorb more calcium and support bone metabolism resulting in a higher bone density and strength (Capcarova *et al.*, 2010).

### 4.2.2.5 Immunomodulation of probiotics

Probiotics can influence the nonimmunologic gut defence by stabilization of the gut flora. In addition probiotic bacteria have been shown to enhance humoral immune response and thereby promote the intestine’s immunologic barrier and also stimulate nonspecific host resistance to microbial pathogens and thus aid in immune elimination, modulate immune response to harmful pathogens with a potential to suppress hypersensitivity
In vitro and in vivo evaluation of health promoting attributes of …

reactions (Isolauri et al., 2001) Probiotics can enhance both specific and nonspecific immune response, by activating the macrophages, and increasing the levels of immunoglobulin (Sanders, 1999). Findings from experimental animals and short term human studies have shown that yoghurt and probiotics such as Lactobacilli and Bifidobacteria stimulate certain cellular and antibody functions of the immune system which in turn may increase resistance to immune related diseases (Wadher et al., 2010). Complex interactions occur between the different constituents of the intestinal ecosystem (resident microflora, epithelial and immune cells) and probiotics. These interactions play a major role in the development and maintenance of immune function linked to the gut associated lymphoid tissue (GALT), including IgA secretion and CD4\(^+\) and CD8\(^+\) T cells activation. It has been indicated by many experiments that changes in the intestinal microbiota can cause immunomodulation at systemic and local levels (Sun et al., 2010).

4.2.2.5.1 Effect of Lactic Acid Bacteria on non specific immune response

Non specific immune response constitutes the first line of defence for the host. It is composed of mononuclear phagocytic cells (monocytes and macrophage), polymorphonuclear leucocytes and Natural Killer cells. The probiotic strains able to survive in the gastrointestinal tract, able to adhere to the intestinal mucous membrane, and able to persist at the critical limit are more effective in stimulating phagocytic cells (Schiffrin et al., 1997). Phagocytosis, a macrophage function is an early and crucial in host defense against invading pathogens. Studies according to Paubert – Braquet et al., 1995; Perdigon et al., 1986 and De Petrino et al., 1995 have shown that the consumption of particular strains of *Lactobacillus* can enhance the phagocytic activity of peritoneal and pulmonary macrophages, the secretion of lysosomal enzyme, and the production of monokines by phagocytic cells.
4.2.2.5.2 Effect of LAB on Lactic Acid Bacteria on specific immune response

The specific immune response could be divided into two main categories, the humoral immunity mediated by antibodies produced by mature B-cells and the cell mediated immunity mediated by T lymphocytes which produce various cytokines and influence other immunocompetant cells (Heric and Levkut, 2002; Tizard, 2000). Secretory immune system plays an important role in the defence mechanism involved in the effective surveillance of mucosal epithelial surface of gastrointestinal tract. It is the predominant isotype in the intestinal secretions of mammals (Heric and Levkut, 2002). IgA is more resistant to the action of proteolytic enzyme that occur in the gastrointestinal secretions (Brandtzaeg et al., 1999). IgA - antigen complexes do not activate the complement with inflammatory outcome. Several Lactic Acid Bacterial strains have been shown to enhance cell mediated immune responses, including T-lymphocyte proliferation, mononuclear cell phagocytic capacity, and tumoricidal activity of Natural Killer cells (Liu et al., 2011; LeBlanc et al., 2002).

4.2.2.6 Antioxidant property of probiotics

Antioxidant activity of Lactic Acid Bacteria have been reported by many studies (Lin and Chang, 2000; Kullisaar et al., 2002; Meira et al., 2012). Oxidative stress and antioxidant potency have been revealed as the key points in molecular regulation of cellular stress responses. Oxidative stress occurs when abnormally high levels of reactive oxygen species (ROS) are generated, resulting in DNA, protein and lipid damage. Living organism have developed an overall antioxidative defense system to mitigate the damaging effects of ROS which include the production of reduced glutathione and enzymes like superoxide dismutase, glutathione, peroxidase and catalase (Kullisnaar et al.}
(2002). Some *Lactobacillus* sp possess antioxidative activity and are able to decrease the risk of accumulation of ROS during the ingestion of food (Kaizu *et al*., 1993; Peuhkuri *et al*., 1996). There are many methods by which the antioxidant property of a probiotic bacteria can be examined. 2,2’-diphenyl-2-picryl-hydrazyl (DPPH) radical accepts an electron or hydrogen radical and gets reduced losing its deep violet colour. DPPH was used for determining the total radical scavenging activity of probiotic bacteria in many studies (Halah and Mehanna, 2011). DPPH radical is known to be stoichiometrically decolorized by potent reducing substance and antioxidants such as cysteine, glutathione, ascorbic acid and tocopherols as reported by Nishino *et al.* (2000).

### 4.2.2.7 Therapeutic application of *Enterococcus faecium*

Enterococci are an essential part of the endogenous gut microbiota of humans and animals and are believed to play a key role in the balance of the microbiota, thereby showing great potential as probiotics (Izquierdo *et al*., 2008). Enterococci have long been used as human and animal probiotics despite the concerns for them as opportunistic pathogens. Although some strains have been implicated as casual agents of antibiotic resistance and human infection, non-pathogenic Enterococci have promising commercial potential (Franz *et al*., 1999). In the European Union 10 preparations of probiotics with 9 different strains of *Enterococcus faecium* have been authorized as additives in feeding stuffs (European Commission, 2004; Foulquie *et al*., 2006). *Enterococcus faecium* SF 68 is the well studied strain of *Enterococcus* used as probiotic. In Denmark, fermented milk product that contains *Enterococcus faecium* SF 68 has been sold for years because of its hypocholesteremic effect (Agerbaek *et al*., 1995). A novel fermented soymilk product with potential probiotic properties was developed by Rossi *et al*., (1999) using the strain *Enterococcus faecium* CRL 183. *In vitro* studies have
revealed a 43% decrease in cholesterol by *Enterococcus faecium* CRL 183 in combination with *Lactobacillus jugurti*. Allen *et al.*, (1996) reported alleviation of symptoms of irritable bowel syndrome in humans by the probiotic strain *Enterococcus faecium* in human clinical trial. Walthers ECOFLOR is a probiotic product that contains *Enterococcus faecium*. The producer of this product claims the efficacy of the *Enterococcus faecium* used in the product against diarrhoea, its anticarcinogenic effect, the production of enterocins active towards *Listeria monocytogenes*, the possible decrease of the LDL-cholesterol level, its sensitivity to Vancomycin and production of lactic acid (Foulquie *et al.*, 2006).

After the introduction and literature review on the potentials of probiotic bacteria in enhancing the host health, the Chapter IV has been divided into two sections. Section 4A which describes the *in vitro* studies on the health promoting effects of *Enterococcus faecium* MBTU-P1F1. Section 4B describes the examination of the health promoting effects *Enterococcus faecium* MBTU-P1F1 through *in vivo* studies.
4A Examination of health promoting properties of *Enterococcus faecium* MBTU-P1F1 (In vitro)

4A.3 Materials and Methods

4A.3.1 Bacterial Strain

A: Test strain - *Enterococcus faecium* MBTU-P1F1

B: β galactosidase negative Lactic Acid Bacteria isolated from Honey bee gut.

C: β galactosidase positive Lactic Acid Bacteria isolated from Honey bee gut.

The strains were isolated in the microbial biotechnology laboratory, School of Biosciences, Mahatma Gandhi University Kottayam, Kerala and maintained as stock culture.

4A.3.2 Examination of health promoting properties of *Enterococcus faecium* MBTU-P1F1 (In vitro)

4A.3.2.1 Resistance to bile salts and bile salt hydrolase activity of *Enterococcus faecium* MBTU-P1F1

4A.3.2.1.1 Resistance to bile salts

Overnight cultures of *Enterococcus faecium* MBTU-P1F1 was inoculated on MRS agar plates supplemented 0.5% sodium salts of the bile salts, sodium taurocholate (SRL laboratories), sodium glycocholate (Sigma) and sodium glycodeoxycholate (Sigma) separately (Vinderola and Reinheimer, 2003). Plates were incubated anaerobically at 37°C for 72 h and observed for growth. The experiment was performed in triplicate.

4A.3.2.1.2 Bile salt deconjugation assay (Ahn et al., 2003)

Overnight culture of *Enterococcus faecium* MBTU-P1F1 was spotted on MRS agar containing 0.5 g/l cysteine and 1mM of the bile salt, sodium taurocholate. Plates were incubated anaerobically at 37°C for 72 h and
observed for precipitation zone around colonies. The experiment was performed in triplicate.

4A.3.2.2 Assimilation of cholesterol by Enterococcus faecium MBTU-P1F1 (Danielson et al., (1989) with modifications)

Cholesterol solution (10 mg/ml) was prepared and filter sterilized. The test medium used for examining the cholesterol uptake ability of the test strain Enterococcus faecium MBTU-P1F1 was sterile modified MRS broth (mMRS) containing 1% glucose and supplemented with bile salt and cholesterol at a final concentration of 100 mg/liter. Two different bile concentrations were used, 0.2 and 0.4% to mimic approximate levels in the intestinal tract. The seed culture was added at a 1% inoculum size to tubes containing 9.9 ml aliquots of the test medium. The cultures were incubated for 12 to 18 h at 37°C under anaerobic conditions. Uninoculated sterile broth was used as the control. Following incubation, bacterial cells were removed by centrifugation, and the spent broth after extraction and uninoculated control broths were assayed for their cholesterol content using cholesterol colorimetric enzymatic assay kit (Agappe Diagnostics) as per the instructions in the manual. The ability of the test strain to remove cholesterol from media was calculated as percentage from the equation:

\[ A=100-\frac{B}{C} \times 100 \]

Where A=% of cholesterol removed, B=absorbance of the sample containing the cells and C=absorbance of the sample without cells. The experiment was performed in triplicate.
4A.3.2.3 Examination of β galactosidase activity of Enterococcus faecium MBTU-P1F1

4A.3.2.3.1 Qualitative test for β galactosidase production

The test was performed using 2-Nitrophenyl β-D-galactopyranoside (ONPG) Test Disks (Sigma). ONPG disc was placed in sterile test tube containing 0.85% NaCl. Colony of the test strain, Enterococcus faecium MBTU-P1F1 was emulsified in the saline. The experiment was performed for the positive and negative control. The tubes were incubated at 35°C and observed at an interval of one hour for 6 h. The experiment was conducted three times.

4A.3.2.3.2 Quantitative test for the determination of β galactosidase activity (Miller, (1998) with modifications)

The experiment was performed with a known positive and negative control. Overnight cultures of Enterococcus faecium MBTU-P1F1, the positive control stain and the negative control strain in MRS broth were centrifuged separately at 12000xg for 5 min at 5°C and washed twice in phosphate buffer pH 7. The cells were inoculated in MRS- lactose broth and incubated for 24 h at 37°C. Cells were harvested and adjusted as OD 0.1 at 560 nm with the buffer. 1ml of the cell suspension of each of the organism was permeabilized with 50 µl Toluene/Acetone (1:9 v/v), vortexed for 7min and analyzed for β-galactosidase activity using Ortho Nitro Phenyl β-D Galacto pyranoside (Sigma). An aliquot (100 µl) of the permeabilized cell suspension was placed in a microtube and 900 µl phosphate buffer and 200 µl of ONPG (2 mg/ml) were added. The microtubes were placed into a water bath at 37°C for 15 min. 0.5 ml of 1M Na₂CO₃ was added to each tube to stop the reaction. The contents of the microtubes were centrifuged at 12000 x g for 5min at 5°C to remove the cells. Absorbance values at both 420 and
560 nm were recorded for each microtubes and β-galactosidase value was calculated in Miller units as following.

\[
1000 \times [A_{420} - (1.75 \times A_{560})] \div \{T \times V \times A_{1560}\}
\]

A1= Absorbance just before assay
A2= Cell density of the reaction mixture

Where T=15 min
V=1ml

The experiment was conducted three times.

**4A.3.2.4 Examination of Antioxidant capability of *Enterococcus faecium* MBTU-P1F1**

DPPH radical scavenging capacity of the test strain *Enterococcus faecium* MBTU-P1F1 was verified using the method of Brand –Williams *et al.*, (1995). A working solution of 60 µmol/l of methanolic solution of DPPH was freshly prepared. 25µl aliquot of cellular suspension of overnight culture of the test strain was transferred to test tubes containing 1ml freshly prepared DPPH solution in eppendorf tubes. The tubes are incubated in dark at 37°C for 45 min. The mixture was centrifuged after incubation and the scavenging activity was measured spectrophotometrically by decrease in absorbance at 517 nm. Blank value was determined by using 1.15g KCL/l. The experiment was conducted with uninoculated MRS broth and ascorbic acid at a concentration of 1mg/ml (positive control).

\[
\% \text{ of scavenging activity} = 1 - \left[ \frac{A_{517\text{nm sample}}}{A_{517\text{nm blank}}} \right] \times 100
\]

The experiment was conducted three times.
4A.4 Results

Probiotic bacteria, especially Lactic Acid Bacteria provide a variety of health benefits. Therapeutic functions attributed to probiotic bacteria are anticholesterol activity, improved lactose utilization, enhancement of immune response and anticarcinogenic activity. The in vitro and the in vivo probiotic characteristics of test strain Enterococcus faecium MBTU-P1F1 was identified in Chapter I and Chapter II, including its colonization capability in Balb/c mice. The part A of the chapter IV (4A) was aimed at examining the health promoting properties of the test strain through in vitro studies. The findings obtained from the experiments are presented here.

4A.4.1 Resistance to bile salts and bile salt deconjugation assay

The test strain Enterococcus faecium MBTU-P1F1 was found to be resistant to sodium taurocholate and sodium glycocholate and sensitive to deoxyglycocholate when grown in MRS media supplemented with these bile salts (Table 4A.1). Moreover fine precipitated white granules were observed around and within the colonies of the test strain grown in MRS supplemented with sodium taurocholate indicating the ability of the strain to deconjugate bile salts and thereby the bile salt hydrolase (BSH) activity of the strain (Fig. 4A.1).

Table 4A.1Resistance of Enterococcus faecium MBTU-P1F1 to bile salts.

<table>
<thead>
<tr>
<th>Bile salts</th>
<th>Enterococcus faecium MBTU-P1F1</th>
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</thead>
<tbody>
<tr>
<td>Sodium taurocholate</td>
<td>Resistant</td>
</tr>
<tr>
<td>Sodium glycocholate</td>
<td>Resistant</td>
</tr>
<tr>
<td>Deoxyglycocholate</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>
4A.4.2 Assimilation of cholesterol

It was observed from the experiment that the test strain had the ability to assimilate cholesterol. As can be learned from Fig.4A.2, the test strain removed 22% of cholesterol from the growth medium supplemented with 0.2% bile salt and 24% of cholesterol in the presence of 0.4% bile salt.

![Cholesterol assimilation](image)

Fig. 4A.2  Cholesterol assimilation ability of *Enterococcus faecium* MBTU-P1F1 expressed as % of cholesterol removal from MRS media containing 0.2 and 0.4% bile salt. Error bar represents the standard deviation of three determinations.
4A.4.3 β Galactosidase activity

The test to detect the β galactosidase property of the test strain was assessed qualitatively and quantitatively using the substrate (ONPG). In the qualitative test, yellow colouration of the filter paper impregnated with the ONPG in saline was observed as a result of contact between the enzyme produced by the test strain and ONPG, indicating that the test strain produces the enzyme β galactosidase. Fig. 4A.3a represents the results. The result obtained was compared with that of the positive and negative control strains. Strong yellow colour was observed in the positive control tube while the negative control tube did not show change in colour.

Further in the qualitative assay the value of the enzyme activity was determined to be 8 Miller Units for the test strain (Fig. 4A.3b). The positive and negative control consisted of LAB isolated from honey bee gut.

Fig. 4A.3a Qualitative analysis using ONPG test disc to examine the production of β galactosidase enzyme by the Enterococcus faecium MBTU-P1F1.
4A.3b Quatitative assay to determine the β galactosidase activity of the Enterococcus faecium MBTU-P1F1 strain in Miller Unit, compared with that of known positive and negative strains.

4A.4.4 Antioxidant property

The DPPH radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants. When DPPH radicals are scavenged the absorbance at 517 nm decreases, as the colour of the reaction mixture changes from purple to yellow. The culture supernatant of test strain exhibited 93.7% DPPH radical scavenging activity. We had used ascorbic acid at 1mg/ml concentration as the positive control. The activity of ascorbic acid was found to be 95% which was almost equivalent to activity of the test strain. The result was also compared with that of uninoculated MRS broth which 50% activity and is summarized in Fig. 4A.4.
4A.5 Discussion

The test strain *Enterococcus faecium* MBTU-P1F1 was tested for its ability to grow in MRS media with the bile salts, sodium taurocholate and sodium glycocholate and deoxyglycocholate. It was found to be resistant to sodium taurocholate and sodium glycocholate and sensitive to deoxyglycocholate. One mechanism of resistance to bile salts could be the ability to deconjugate bile salts and this capacity has been related to the capacity to remove cholesterol from the intestinal environment (Begley et al., 2005; Vinderola et al., 2008). Bacteria producing bile salt hydrolase have the ability to deconjugate bile salts and this activity play role in gut microflora equilibrium and is considered as an important colonization factor.
BSH activity contributes to resistance to the toxicity of conjugated bile salts in the duodenum. BSH enzyme might be a detergent shock protein that enables a probiotic strain to survive the intestinal stress (De Smet et al., 1995). The BSH activity is yet another desirable property which can be considered while selecting a strain to be used as dietary adjuvant. The Bile salt hydrolase activity is also considered as a mechanism to reduce cholesterol. Deconjugated bile salts are less soluble and get easily excreted in the feces. In order to replenish the bile salts, more molecules of cholesterol (the precursor of bile salt) are spent. Thus more excretion of deconjugated bile salts, lead to increased synthesis of bile salts which in turn lead to removal of cholesterol from body (Djouvinov et al., 2005). Furthermore, deconjugated bile salts do not stimulate the absorption of cholesterol and other lipids from the small intestine as well as do conjugated bile salts.

Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease. Although therapeutic drugs are available to relieve this problem, they are often expensive and have side effects. It has been indicated that Lactobacillus sp. was able to reduce cholesterol through several mechanisms including bile salt deconjugation (Sieladie et al., 2011). Ability of the test strain to assimilate cholesterol was examined in modified MRS broth supplemented with two different concentrations of bile salts. Our findings revealed the cholesterol lowering property of the test strain Enterococcus faecium MBTU-P1F1. Probiotic strains assimilate cholesterol for their own metabolism. Liong and Shah, (2005) reported that a portion of the cholesterol assimilated by Lactobacillus strains was incorporated into the cellular membrane. They bind to the cholesterol molecule, degrading it to its catabolic products. Increase in the
percentage of removal of cholesterol by the test strain with increase in the concentration of bile salt was observed in the study. Increased uptake of cholesterol in presence of more bile salts is due to the co-precipitation of cholestetol with deconjugated bile salts. Similar finding was observed Pereira and Gibson, (2002). Probiotic strains are known to reduce cholesterol indirectly by deconjugating the cholesterol to bile acids and thereby reducing the body pool (Sudha et al., 2009). Zhu et al., 2009 reported that Enterococcus faecium X5 and Enterococcus faecium Y5 showed higher ability to lower cholesterol with 35.49 and 33.63% of cholesterol lowering rate respectively. Interestingly we observed 24% cholesterol lowering property for the test strain in the presence of 0.4%. The result also supports the finding that the test strain exhibits bile salt hydrolase activity. Furthermore we have examined the cholesterol lowering ability of the strain in Balb/c mice.

It was found in the quantitative and qualitative study that $\beta$ galactosidase activity was present in the test strain Enterococcus faecium MBTU-P1F1. $\beta$ galactosidase production is one of the few well established probiotic effects of LAB identified to date (Charteris et al., 1998). The enzyme is widely used in dairy industry and is produced by most Lactobacilli. The enzyme hydrolyses lactose in milk into glucose and galactose, which can be absorbed across the intestinal epithelium (Vasiljevic and Jelen, 2001). The test strain Enterococcus faecium MBTU-P1F1 produces the enzyme $\beta$ galactosidase with a value of 8 Miller units activity. Vinderola and Reiheimer, (2003) observed that the value of $\beta$ galactosidase activity was ranged from 0-2053 Miller units among various probiotic strains of Lactobacillus delbruecki subsp. bulgaricus. Also absence or low values of the enzyme were reported in the probiotic bacteria Lactobacillus casei and
*Lactobacillus rhamnosus.* Lactose intolerance is due to the insufficient activity of the enzyme lactase in the human gut causing abdominal discomfort. The symptoms of lactose intolerance can be reduced by eliminating lactose from the diet or by using of supplemental $\beta$-galactosidase enzyme replacement (Vasiljevic and Jelen, 2001). Lactic Acid Bacteria is one of the important microbial sources of $\beta$-galactosidase due to their GRAS status and according to WHO guidelines for an ideal probiotic, the ability to produce $\beta$ galactosidase is considered mandatory. Addition of bacteria capable of producing the enzyme as probiotic to infant formula could improve lactose digestion and thus helps to reduce lactose intolerance symptoms. The discovery of new strains producing high level of the enzyme has gained importance for potential application in probiotic cultures. It can be concluded from the discussion that the $\beta$ galactosidase activity of the test strain enhances its quality as a probiotic strain.

The DPPH radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants. When DPPH radicals are scavenged the colour of the reaction mixture changes from purple to yellow and the absorbance at 517 nm decreases accordingly. We examined the antioxidant property of the test strain *Enterococcus faecium* MBTU-P1F1 by determining the free radical scavenging activity of cells grown in MRS medium. Oxidation is a needed reaction in an organism’s metabolism which can also lead of production of ROS. High levels of reactive oxygen species (ROS) will cause damage to host’s DNA, proteins, lipids and carbohydrates. Current research in the antioxidant properties of LAB has shown that LAB strains and their metabolic products reduce the risk of ROS accumulation through food ingestion, and also degrade superoxide anion and hydrogen peroxide (Liu *et al*., 2011).
Remarkable scavenging activity was observed for the test strain indicating the production of antioxidants by the strain. LAB exert antimicrobial action through various mechanisms for example *Lactobacillus casei* can cause a decrease of malondialdehyde (MDA), and increase of superoxide dismutase (SOD) and glutathione peroxidise (GSH-Px) in serum and liver (Zhang and Zhang, 2013). Interestingly the scavenging activity (93%) exhibited by the test strain corresponded to the antioxidant activity of 1mg/ml ascorbic acid. Studies of extracellular polysaccharides from LAB have been focused on antioxidant polysaccharides with potential applications in the food industry (Kishk and Al-sayed, 2007). Much attention has been paid to microbial exopolysaccharide for their potential therapeutic activities including antioxidant properties. Antioxidant effect of exopolysaccharide isolated from the marine yeast *Meyerozyma guilliermondii* have been proved by Koilery, K. S. 2013, Ph.D. thesis, Mahatma Gandhi University. Presence of cell surface polysaccharides of the test strain has been already proved in chapter II under cell surface properties. It has been reported by Nedelcheva et al., (2010) that the application of probiotic bacteria with antioxidant activity in foods increases their biological activity, quality, as well as their shelf life.
4B Examination of health promoting properties of Enterococcus faecium MBTU P1F1 (In vivo)

4B.3 Materials and methods

4B.3.1 Animal model

Male Balb/c mice (8 week old, (20±0.8g) were used in the study. Balb/c mice were obtained from small animal breeding station under Kerala Agricultural University, Department of Veterinary and Animal Sciences, Thrissur, Kerala. The animals were kept in the animal house of School of Biosciences, Mahatma Gandhi University Kottayam, Kerala. Animals were housed in polypropylene cages and were given standard sterile dry pellet (Sai Feeds, Bangalore, India) and sterile drinking water ad libitum. The animals were maintained at a controlled condition of temperature of 26-28°C with a 12 h light: 12 h dark cycle. Bedding in cages was changed every day. Care and use of animals under study were followed according to the institutional guidelines of Mahatma Gandhi University.

4B.3.2 Preparation of inoculum for in vivo study

Enterococcus faecium MBTU-P1F1 was cultured in MRS for 24 h at 37°C in the presence of 5% CO₂. The culture was centrifuged at 1500 g for 10 min at 4°C and washed with PBS thrice and re suspended in sterile PBS. For assessment of the number of viable bacteria, suitable dilutions of the bacterial suspensions were plated onto MRS agar plates and no of colony forming units (CFU) were counted after 24 h at 37°C. The dilution of the culture supernatant containing 10⁸ viable cells per ml was chosen and turbidity was measured spectrophotometrically at 560 nm.
4B.3.3 Experimental design for evaluation of \textit{in vivo} studies

Mice assigned for each set of experiment was divided into 2 groups, comprising of one test group and one control group with 6 mice each. The two groups were caged separately and were given autoclaved food and water. Beddings of the cages and water bottles were cleaned and changed each day. Experimental design for the study is summarized in Table 4B.1.

\begin{table}[h]
\centering
\caption{Experimental design and grouping of animals}
\begin{tabular}{|l|c|c|}
\hline
Experiment & No. of Mice in Test Group & No. of Mice in Control Group \\
\hline
EXPT-1 Haemolytic and Biochemical parameters. Antioxidant property of liver & 6 & 6 \\
EXPT-2 Humoral Immune Response & 6 & 6 \\
EXPT-3 Cell mediated Immune Response. Splenic lymphocyte proliferation & 6 & 6 \\
EXPT-4 Delayed Type hypersensitivity & 6 & 6 \\
\hline
\end{tabular}
\end{table}

4B.3.4 Oral treatment

The mice in the test group were given daily oral administration of 1ml ($10^8$ viable cells) of the bacterial suspension in sterile PBS by orogastric gavage with a ball tip needle. The control group received 1ml of PBS alone. The treatment was given consecutively for 20 days.

4B.3.5 Evaluation of the effect of oral administration of \textit{Enterococcus faecium} MBTU-P1F1 on Haematological and Biochemical parameters of Balb/c mice

4B.3.5.1 Collection of blood from experimental animals (Balb/c mice)

Blood samples from each individual mouse assigned for EXPT -1 (test and control groups) were collected with, a capillary pipette containing
Chapter IV

anticoagulant EDTA. The pipette was inserted in the lateral canthus and blood was collected from the retro orbital sinus. After collection, the pipette was removed and bleeding stopped when the eye returned to a normal position. The collected blood was divided into two parts. The blood plasma was separated from one part by centrifugation at 3000 g for 30 min.

4B.3.5.2 Collection of serum from experimental animals

Blood was collected from retro orbital sinus into a 1.5 ml vial. It was allowed to stand for 2 h at room temperature, then centrifuged at 12,000g for 15 min at 4°C to separate the serum. Serum collected from all the animals in the test and control groups were stored at -20°C for analysis.

4B.3.5.3 Determination of total red blood cell (RBC) count

RBC count was determined in Neubauer chamber using whole blood as described by Chaudhari, 2000a.

Total RBC count = Number of cells counted × 10,000 count/mm$^3$

4B.3.5.4 Determination of total white blood cell (WBC) count

(WBC count was determined in Neubauer chamber using whole blood.

Total number of WBC = Number of cells counted × 50 count/mm$^3$

4B.3.5.5 Determination of haemoglobin (Hb) in blood (Drakin and Austin, 1932)

The study was conducted by using Agappe diagnostic kit for determination of Hb in blood according to the manufacturer’s instruction.

\[
\text{Haemoglobin (g/dl)} = \frac{\text{OD}_T \times 60 \times 0.251}{\text{OD}_S}
\]

\(\text{OD}_T\) = Optical Density of test at 546 nm

\(\text{OD}_S\) = Optical Density of standard at 546 nm
4B.3.5.6 Determination of Mean corpuscular volume (MCV), Mean corpuscular haemoglobin concentration (MCHC)

Blood sample was taken in small sterile centrifuge tube and centrifuged at 1500 rpm for 10 min at 4°C. RBC layer was separated and the above parameters were determined with an automated haemoanalyzer.

4B.3.5.7 Determination of biochemical parameters

Evaluation of the biochemical parameters was based on the energy profile and the mineral profile. The energy profile included the total lipid profile and protein level in serum of Balb/c while mineral profile included examination of calcium and inorganic phosphorus in serum. The biochemical parameters were analyzed using semi automated clinical chemistry analyzer.

4B.3.6 Evaluation of the immunomodulatory effect of oral administration of Enterococcus faecium MBTU-P1F1 in Balb/c mice

4B.3.6.1 Effect on Humoral Immune Response

4B.3.6.1.1 Collection of serum and intestinal fluid

Serum from the animals was collected as under section 4B.3.5.2. The animals were sacrificed and the intestine was separated. The contents in the intestine were removed and discarded. The intestine is cut open and washed with PBS to remove residual food particles. The wall of the intestine was scraped using PBS containing 1ml of 0.5% Tween 20. Washed small intestine was disrupted with a glass homogenizer and mixed with four fold volume of the buffer. The suspension is centrifuged at 10,000g for 15 min and the supernatant obtained used as the tissue extract of the small intestinal wall for the detection of intestinal Ig A response and is measured by ELISA.
4B.3.6.1.2 Enzyme linked immunoabsorbent assay to determine serum immunoglobulin level and secretory IgA

Serum antibody levels of IgA, IgE, IgG, and IgM and the level of Ig A in the intestinal fluid were analyzed using the mouse IgA, IgE, IgG, and IgM ELISA quantitation kit. Serum antibody levels of all groups under study were assayed according instructions on the Quantitation Kits (Mouse Ig G ELISA kit catalog number: E-90G Mouse Ig M ELISA kit catalog number: E-90M, Mouse Ig A ELISA kit catalog number: E-90A, Mouse Ig E ELISA kit catalog number: E-90E CRL Laboratories). Diluted serum samples for enzyme assay were prepared according to the specific kit instructions. Briefly, IgA, IgE, IgG, and IgM levels in test serum’s were measured by sandwich enzyme-linked immunoabsorbent assay (ELISA). The microtiter plate provided in kit has been pre-coated with antibody specific to immunoglobulins. Diluted serum test samples and standards were added to predetermined wells in microtiter plates. All reagents were brought to room temperature before use. Pipette 100 µl of standards in various concentrations and test sample in duplicate into predesignated wells. The microtiter plate was incubated at room temperature for 30 min. Plates were kept covered during incubation. After incubation, the contents of the wells were aspirated. Each well was completely filled with appropriately diluted wash solution and aspirated. Plate was inverted and residual buffer was removed by sharply striking on absorbent paper. After a total of four washes 100 µl of appropriately diluted Enzyme-Antibody Conjugate was added to each well and incubated at room temperature in dark for thirty minutes. Wells were washed and blotted. 100 µl of TMB substrate solution was added into each well. The plate was incubated in the dark at room temperature for precisely 10 min. After ten min, 100 µl of stop solution was added to each well. The absorbance was determined at 450 nm with the contents of each well. The concentration of immunoglobulins in the samples
was then determined by comparing the O.D. of the samples to the standard curve. Equivalent levels of test immunoglobulins were calculated by comparing the levels with a reference curve generated using immunoglobulin standards. Results were expressed according to the concentrations of each immunoglobulins in serum.

4B.3.6.1.3 Hemagglutination antibody titer

Principle

The non-agglutinated Sheep red blood cell (SRBC) will settle to the bottom of the well as a clear button while agglutinated cells will form a diffused mat. The titre of the antibody was determined as the reciprocal of the highest dilution of serum at which a clear agglutination was observed.

4B.3.6.1.3.1 Preparation of SRBC

Fresh sheep blood was collected from the local slaughterhouse in vials under sterile conditions in sterile freshly prepared Alsevere’s solution in a 1:1 proportion and stored in refrigerator. Red blood cells were separated, by centrifugating at 2000 rpm for 10 min. The cells were washed with PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH – 7.4) 4-5 times and were suspended in PBS to get a final concentration of $1 \times 10^8$ cells/ml for immunization and challenge.

4B.3.6.1.3.2 Sensitization of Balb/c mice with SRBC

The animals in the test and control groups belonging to EXPT-2 were immunized by intra peritoneal administration of 0.1 ml of $1 \times 10^8$ SRBC/mouse. The day of immunization was considered as day 0.

4B.3.6.1.3.3 Assessment of antibody titer (Puri et al., 1994)

To determine the antibody response to SRBC, a direct hemagglutination technique was used. On day 1, blood samples were
collected and serum was separated as previously described under section 4B.3.5.2 from all test and control animals in EXPT-2.

Two fold serial dilutions of individual serum samples were made with phosphate buffered saline in microtitre plate to get final volume of 25 µl. The control well contained only PBS. 25 µl of SRBC prepared in section 4B.3.6.1.3.1, was added to all wells for a final volume of 50 µl. The plates were then shaken for 1 min and incubated at 37°C for 1 h to determine agglutination titre. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

4B.3.6.2 Cell mediated immune responses

4B.3.6.2.1 Effect of Enterococcus faecium MBTU-P1F1 on phagocytosis by peritoneal macrophages in Balb/c mice

Phagocytosis is the process by which phagocytic cells ingest particles whose size exceeds about 1µm and this phenomenon is a critical event in innate immune response. Complex signaling pathways promoted by the engulfment of targets lead to fusion of protease-rich granules with the phagosome. This can trigger oxidative burst and finally killing of the pathogen. The effect of the test strain on the phagocytic activity of the peritoneal macrophages was examined by two methods. The experiment was performed with the test and control group mice assigned in EXPT-3.

4B.3.6.2.1.1 Isolation and preparation of peritoneal macrophage cell suspension (Ray et al., 2010)

After oral treatment of mice with Enterococcus faecium MBTU-P1F1 for 20 days, each mouse belonging to EXPT-3 was euthanized, sprayed with 70% ethanol and mounted on the styrofoam block on its back. By using a scissors and forceps a cut was made on the outer skin of the peritoneum and gently pulled it back to expose the inner skin lining of the peritoneal cavity.
2 ml of ice cold PBS was injected into the peritoneal cavity using a 27g needle. The needle was slowly pushed into the peritoneum being careful not to puncture any organs. After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. A 25 g needle, attached to a 5 ml syringe was inserted into the peritoneum and the fluid was collected while moving the tip of the needle gently to avoid clogging by the fat tissue or other organs. The collected cell suspension was deposited in tubes kept on ice after removing the needle from the syringe. An incision was made in the inner skin of the peritoneum and while holding up the skin with a forceps, the remaining fluid was collected from the cavity by the use of plastic Pasteur pipette and transferred in collection tube kept in ice. The collected cell suspension was centrifuged at 1500 rpm for 8 min, at 4°C and the supernatant was discarded and the cells were resuspended in PBS for counting. Trypan blue exclusion was used to check the viability of the immune cells. The cell suspension and 1% trypan blue solution were mixed in 1:1 proportion. Cells were loaded into hemocytometer and the number of stained and unstained cells was counted in the hemocytometer. The peritoneal cell suspension of macrophages obtained from each mice belonging to the test and control group was diluted with PBS to contain uniform number of viable cells in all samples.

4B.3.6.2.1.2 Phagocytosis Evaluation (Wadekar et al., 2008)

0.1 ml of the macrophage cell suspension was smeared onto a clean sterile glass slide. The slide was kept in a moist cotton pad in a sterile chamber and was incubated at 37°C for 20 min in the presence of 5% CO₂ for facilitating the attachment of cells to the glass slide. After incubation, the slide was slowly drained with sterile Hanks balanced salt solution (HBSS) taking care not to wash off the adhered macrophages. The slide was then
flooded with a suspension of heat killed yeast cells and was incubated at 37°C for one hour in the presence of 5% CO₂. Yeast suspension was drained with PBS and the slides were fixed in absolute ethanol for 2 min. Then it was dried and stained with Giemsa stain. 100 random macrophages and the number of yeast cells phagocyntosed by each macrophage were counted using oil immersion (100X objective). The value obtained was taken as phagocytic index (PI). The percentage of phagocytosis (immune stimulation) was calculated from the PI of the test group and control group using the following equation,

\[
\text{% of phagocytosis} = \left[ \frac{\text{PI (test)} - \text{PI (Control)}}{\text{PI Control}} \right] \times 100
\]

4B.3.6.2.1.3 Nitroblue tetrazolium Assay (NBT) (Freeman and King, 1971) Principle of the assay

In this test, we measure the respiratory burst, the intracellular killing by oxidative mechanism by macrophages. Upon activation, macrophages are highly effective at generating reactive oxygen species (ROS) by a process known as the respiratory burst. NADPH oxidase of macrophages facilitates the transfer of one electron from cytosolic NADPH to molecular oxygen captured from the medium. The product of this reaction, the superoxide anion (O\(^{-}\)), can then be converted to other reactive oxygen metabolites, including hydrogen peroxide and hypochlorous acid. Nitro blue tetrazolium (NBT) is an electron acceptor used to detect indirectly the production of superoxide by stimulated macrophages. Superoxide reduce the yellow soluble NBT to blue black formazan, an insoluble material that precipitate and can be seen microscopically within the cell. Hence the test can be used as a qualitative assessment of superoxide production.
Preparation of Nitroblue tetrazolium (NBT) dye

0.1% stock solution NBT was prepared using commercially available, NBT in saline. Gentle heating was required to dissolve the NBT. Working solution was made by mixing 1 ml of the stock solution with 1 ml phosphate-buffered saline. The solution was freshly prepared before the test.

Procedure

For the assay, 0.1 ml peritoneal cell suspension prepared under section 4B.3.6.2.2 was incubated with 0.1ml NBT for 30 min at 37°C with 5% CO₂. After incubation, a drop from each sample was carefully transferred onto a grease free glass slide and a cover slip smear was made. The smear was allowed to dry and fixed with ethanol for 3 min. Then, the slides were stained with saffranin for 3 min air dried and observed under 40X objective. 100 macrophages with formazan granules were counted for calculating percentage of macrophages.

4B.3.6.2.2 Delayed type hypersensitivity (Karthik Kumar et al., 2011)

Principle

T cells are immune response mediators and play an important role in establishing and maximizing the capabilities of the adaptive immune response. By using SRBC the stimulation of the T-lymphocyte activity was checked by measuring the foot pad swelling.

Procedure

The test was performed with the test and control group mice assigned in EXPT-4

The animals in both groups were immunized by intra peritoneal administration of 0.1 ml of 1x10⁸ SRBC/mouse. The day of immunization was considered as day 0. On the day 1 (24 h after immunization), animals in all the
groups were challenged by subcutaneous administration of 0.2 ml of SRBC 1% into the right hind foot pad, while a 0.2 ml of PBS was administered in the left hind foot pad to serve as control. DTH response was measured at 48 h after the immunization (day 2) and expressed as percent increase in paw volume. The inflammation percentage was calculated as per Manosroi et al., 2005.

\[
\text{Inflammation (\%)} = \frac{(\text{FPS after treatment} - \text{FPS before treatment})}{\text{FPS before treatment}} \times 100
\]

**4B.3.6.2.3 Proliferation of splenic lymphocytes** (Bujalance et al., 2007)

**Principle**

The Methyl Thiazolyl Tetrazolium (MTT) Cell Proliferation Assay measures the cell proliferation rate. Assay is based on the ability of a mitochondrial dehydrogenase enzyme (by generating reducing equivalents such as NADH and NADPH) from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals, which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

**Isolation of splenocytes** (Shalini et al., 2009)

The test was performed with the peritoneal cell suspension of each mouse from the test and control group in EXPT-5.

Animals of the test group and the control group were sacrificed by cervical dislocation and the skin underlying the peritoneal region was removed and spleen was collected aseptically. The spleens were teased apart in cold Hank’s balanced salt solution (HBSS) with a sterile needle and forceps. The teased cells were transferred to a sterile centrifugation tubes. Clumps were allowed to settle in a centrifugation tube kept in ice for 20 min.
Supernatants were collected in separate tubes and resuspended in sterile RPMI 1640 media (Himedia) with HEPES buffer (20 mM), sodium bicarbonate (24 mM) and Gentamicin (100 µg/ml). 1 ml upper portion of the medium containing the splenocytes were aseptically transferred using micropipette to a 15 ml sterile centrifuge tube. Tube was kept to stand in ice for 2 min. After that cell suspensions were transferred to fresh centrifuge tube and centrifuged at 1000 g for 5 min, at 4°C. Cells were washed once again in basal culture medium and then 1ml of erythrocyte lysis buffer were added to the pellet. After 45 S, 5 ml of culture medium was added and centrifuged at 1000 g for 5 min. Lymphocytes were washed twice with culture medium to remove the traces of lysis buffer and finally suspend in 1 ml of RPMI 1640 containing 10% Fetal Calf Serum (FCS). Trypan blue exclusion was used to check the viability of the immune cells.

**Procedure**

Proliferation of spleen cells were measured by methyl thiazolyl tetrazolium (MTT) incorporation as described by Buja-lance with minor modifications. Effect of the mitogens Concanavalin A (T cell) and lipopolysaccharide (B Cell) on lymphocyte proliferation was analyzed. Cell suspension containing 1x10^7 viable cells/ ml was made in RPMI-1640 (Himedia) containing 10% FCS (Section 4B.3.6.2.6). 100 µL of cell suspension were dispensed into 96-well flat bottomed tissue culture plates. Cells were allowed to grow in the presence of 10 µL mitogen (concanavalin A (1.5 µg/ml ) and lipopolysaccharide (5 µg/ml)) of the mitogens separately to determine the difference between cell proliferation. Plates were incubated in a humidified CO₂ incubator under 5% carbon dioxide at 37°C for 72 h. Methyl thiazolyl tetrazolium (SRL Laboratories) was dissolved in RPMI-1640 (Himedia) at 5 mg/ mL concentration and filter sterilized through 0.22 µm
filters. 10 µL of MTT was added to each well, and the plates were incubated at 37°C for 4 h. During this period, formazan crystals will be formed at the bottom of each well. 70 µL of supernatant was removed from each well and discarded; Cell dissociation solution (20% wt/vol sodium dodecyl sulfate, 50% vol/vol dimethyl sulfoxide, pH 4.5) was added to each well and incubated overnight at 37°C. Spleen cell proliferation was measured by determining the absorbance at 570 nm using an automated microplate reader (Elisa plus). Proliferation ratio, was calculated according to the following equation:

\[
\text{proliferation ratio} = \frac{(\text{test OD570} - \text{control OD570})}{\text{control OD570}} \times 100\%,
\]

where OD570 is the optical density measured at 570 nm.

**4B.3.7 Effect of oral administration of Enterococcus faecium MBTU-P1F1 on the antioxidant status of liver**

Livers of mice in the control and test group from EXPT-4 were excised, cleaned and homogenized in 1:5 volumes of normal saline solution. The homogenate was centrifuged at 1700×g for 10 min at 4°C. The supernatant fractions were collected and antioxidant activity was determined based on DPPH radical scavenging activity by spectrophotometric methods as per Brand-Williams et al., (1995) with modifications. A working solution of 60 µmol/l of methanolic solution of DPPH was freshly prepared. Transfer 10 µl of the supernatant fractions from the liver homogenate of the control and treatment mice into respective wells in a 96 well microtitre plate. Add 80 µl of DPPH working solution to each of the wells. Cover the plate with aluminium foil and incubate in dark for 120 min at 37°C. Ascorbic acid at a concentration of 1mg/ml was used as positive control. Blank value was determined by using 1.15g KCL/l. Absorbance at 517 nm was measured and the percentage of scavenging activity was calculated as –

\[
\% \text{ of scavenging activity} = 1 - \left[ \frac{A_{517\text{nm sample}}}{A_{517\text{nm blank}}} \right] \times 100
\]
4B.3.8 Statistical Analysis

The statistical significance of each test group in comparison to control animals was tested at the P=0.05 level using one tailed t-test.

4B.4 Results

Based on the results from Chapter II, it was evident that gastrointestinal colonisation in Balb/c mice by the test strain could be achieved after oral administration of the cell suspension of the test strain in PBS ($10^8$ CFU/ml) for 20 days. Therefore the mice in the test group were given similar treatment and those in the control group received 1ml of PBS.

4B.4.1 Evaluation of the effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the haematological parameters in Balb/c mice

In order to study the effect of test strain on the general health of the host animal, we examined the haematological parameters such as, Haemoglobin %, MCV, MCHC, RBC, WBC and the differential count of WBC. It was described in chapter II that oral administration of the test strain did not have any adverse effect on the general health of the test animal group. The haematological studies further supported these finding and no significant changes in the various blood parameters were observed compared to the control mice group. The values obtained for the test group and control group for haematological parameters are shown in Table 4B.1.
Table 4B.1 Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the haematological parameters in Balb/c mice *

<table>
<thead>
<tr>
<th>Haematological Parameters</th>
<th>Test Group-Recieved <em>Enterococcus faecium</em> MBTU-P1F1 as probiotic for 20 days</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (gm %)</td>
<td>16 \pm .6</td>
<td>15.5 \pm .4</td>
</tr>
<tr>
<td>MCV (%)</td>
<td>48 \pm 1</td>
<td>49 \pm .8</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>31 \pm 0.5</td>
<td>32 \pm 0.8</td>
</tr>
<tr>
<td>RBC (million/cumm)</td>
<td>5.1\pm 0.37</td>
<td>5.1 \pm 0.25</td>
</tr>
<tr>
<td>WBC (cumm)</td>
<td>5,050 \pm 60</td>
<td>5,000 \pm 48</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>21\pm 2.37</td>
<td>20 \pm 1.49</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>75\pm 3.71</td>
<td>74.7 \pm 2.32</td>
</tr>
<tr>
<td>Monocytes %</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Eosinophils %</td>
<td>0.83\pm 0.01</td>
<td>1</td>
</tr>
<tr>
<td>Basophils %</td>
<td>0.810+/-.02</td>
<td>0</td>
</tr>
</tbody>
</table>

*P >0.05 (test group vs. test group). WBC: White Blood Cells, RBC: Red Blood cells, MCV: Mean Corpuscular Volume, MCHC: Mean Corpuscular Hemoglobin Concentration.

4B.4.2 Evaluation of the effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the biochemical parameters in Balb/c mice

Study of the biochemical parameters included the energy profile and mineral profile. The results are presented in Table 4B.2. Significant reduction in the concentration of serum triglycerides was observed in the test group, compared to the control group. Also we found reduction in serum cholesterol level of the test group with HDL level more than the LDL. It can be learned from the results that the mice belonging to the test group have a healthy serum lipid profile with low cholesterol level and low triglyceride concentration than the control group. No significant difference in the serum protein level of the test group and the control group was observed. Mineral
profile based on the serum level of calcium and inorganic phosphorus revealed increase in their levels compared to the control group.

**Table 4B.2** Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the Energy Profile and Mineral Profile in Balb/c mice

<table>
<thead>
<tr>
<th>Energy Parameters</th>
<th>Test Group receiving <em>Enterococcus faecium</em> MBTU-P1F1 as probiotic</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Cholesterol (mg/dl)</td>
<td>75.0 ± 3*</td>
<td>88.0 ± 3.8</td>
</tr>
<tr>
<td>Serum Triglycerides (mg/dl)</td>
<td>147.0 ± 4.3*</td>
<td>266.0 ± 9.2</td>
</tr>
<tr>
<td>Serum HDL (mg/dl)</td>
<td>45.0 ± 2</td>
<td>43.0 ± 1.8</td>
</tr>
<tr>
<td>Serum LDL (mg/dl)</td>
<td>19.4 ± 1.9</td>
<td>25.0 ± 2</td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
<td>6.30 ± 0.8</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>13.3 ± 0.5</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>Phosphorous (mg/dl)</td>
<td>10.8 ± 0.7</td>
<td>8.6 ± 0.2</td>
</tr>
</tbody>
</table>

*P < 0.05 (test group vs. control group).

4B.4.3. Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the immune response of the host animal

4B.4.3.1 Humoral immune response

4B.4.3.1.1 Serum immunoglobulin levels

Role of the test strain on the humoral immune response of the host was assessed by examining the level of immunoglobulins in the serum. Significant increase (p < 0.05) in the level of the immunoglobulins IgM, and IgG was obtained as can be learned from Table 4B.3. The level of the allergic antibody IgE, was found to be 3.01 IU/ml and fell within the physiological range. Concentration of the serum Immunoglobulin IgA was found to be low in the test group than the control group but the value was again within the physiological range. It was observed that the level of the Secretory IgA showed only a slight increase in the intestinal fluid than the control group and the difference was not statistically significant (P>0.05).
Table 4B.3  Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the humoral immune response in Balb/c mice

<table>
<thead>
<tr>
<th>Name of Group</th>
<th>Serum immunoglobulins</th>
<th>Secretory IgA</th>
<th>Haemagglutination Titer Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Group-Oral treatment of <em>Enterococcus faecium</em> MBTU-P1F1 for 20 days</td>
<td>IgG: 5.87* ± 0.76</td>
<td>IgA: 0.2 ± 0.02</td>
<td>IgM: 1.37* ± 0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgE: 3.01 ± 0.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Group</td>
<td>IgG: 1.25 ± 0.34</td>
<td>IgA: 0.97 ± 0.07</td>
<td>IgM: 0.86 ± 0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgE: 2.1 ± 0.83</td>
</tr>
</tbody>
</table>

*P<0.05 (test group vs. control group)

4B.4.3.1.2 Haemagglutination assay

The effect of the test strain on the humoral immune response was also studied by examining the level of antibody produced towards SRBC in the serum of the test group and control group. It can be read from Table 4B.3 that the antibody titre of the test group mice was higher than the control group animals indicating that the oral administration of the test strain can enhance specific responsiveness of the host towards antigens. Statistical analysis showed that the difference between the antibody titre of the test group and the control group is significant with P<0.05.

4B.4.3.2 Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on cell mediated immune response in Balb/c mice

4B.4.3.2.1 Phagocytic activity and NBT assay of peritoneal macrophages

Considerable increase in the phagocytic activity of the peritoneal macrophages was observed in the test group which received *Enterococcus faecium* MBTU-P1F1 as probiotic for 20 days. The phagocytic index was
determined by counting the number of yeast cells phagocytosed by the macrophages in 100 microscopic fields. Percentage of stimulation of immune response of the test group with respect to the control group was calculated from the phagocytic index of the test group mice and the control group. The results are given in Table 4B.4 and Fig. 4B.1.

**Table 4B.4** Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on the phagocytic activity of peritoneal macrophages

<table>
<thead>
<tr>
<th>Mice Group</th>
<th>Phagocytic index</th>
<th>% of stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Group</td>
<td>170+/-15</td>
<td>33%</td>
</tr>
<tr>
<td>Control Group</td>
<td>132+/-9</td>
<td>-</td>
</tr>
</tbody>
</table>

The enhancement of phagocytosis by the test strain was further substantiated by the NBT assay. The percentage of cells reducing the electron accepter NBT was used as a measure of the respiratory burst exhibited by the macrophages. Significant enhancement (P<0.05) in the number of cells reducing NBT was observed in the test group which received $10^8$ viable cells of the test strain for 20 days. The result is represented in Fig. 4B.2.
Fig. 4B.2  Effect of oral administration of Enterococcus faecium MBTU-P1F1 on phagocytic activity by NBT Assay.

Fig. 4B.3a  Delayed Type Hypersensitivity represented as % of inflammation of foot pad
4B.4.3.2 Delayed type hypersensitivity

The increase in the sole thickness of mice in the test and control group was measured 24 h after the 2nd inoculation of SRBCs. The extent of local inflammation determined by the increase in the sole thickness was used to measure the effect of oral administration of the Enterococcus faecium
MBTU-P1F1 on delayed type hypersensivity. Highly significant increase (P<0.05) in the percentage of inflammation was observed in the test group mice compared to the control group mice. The Fig. 4B.3a, 4B.3b and 4B.3c demonstrates the results.

4B.4.3.2.3 Proliferation of splenic lymphocytes

The proliferative response of splenocytes was estimated using the colorimetric MTT assay using the mitogens Concanavalin A and lipopolysaccharide. The effect of the test strain on splenic lymphoproliferation are shown in Fig. 4B.4. As can be learned from the data, we observed only slight increase in the proliferation of the splenic lymphocytes in the test group when compared to the control group. Also the proliferative response was more towards the T cell mitogen concanavalin A than towards B cell mitogen lipopolysaccharide. However the differences observed between the test group and the control group were not statistically significant with P>0.05.

**Fig. 4B.4.** Effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on splenic lymphocyte proliferation in Balb/c mice
4B.4.4 Antioxidant assay in liver

Effect of the test strain on the antioxidant property of the liver was assayed based on the ability to reduce the DPPH radical. The DPPH radical scavenging activity of the liver tissue was more for the test group mice than the control group and difference was significant with P<0.05. Ascorbic acid at 1mg/ml concentration was used as the positive control (Fig. 4B.5).

![Antioxidant activity of Liver](image)

**Fig. 4B.5** Antioxidant activity of liver

4.5 Discussion

Part B of the chapter IV evaluated the health promoting capabilities of *Enterococcus faecium* MBTU-P1F1 in Balb/c mice. Persistence studies as well as pathogenicity assessment of oral administration of the test strain as probiotic were established in Chapter II under the sections 2.4.6.2 and 2.4.6.3 and discussed under the section 2.5.
Changes in haematological parameters from the normal physiological range are regarded as an indication of disease condition. The safety of oral administration of the test strain in the study as a probiotic has already been discussed in Chapter II. The result obtained from the present test further substantiates the safety of the test strain for use as a potential probiotic. No significant changes were observed for any of the haematological parameters tested including in the differential WBC count indicating absence of infection. Many researchers have analyzed the effect of probiotic administration on the haematological parameters. Our result is in same line with the findings of Djouvinov et al. (2005) that blood parameters of mule ducklings were not influenced by the probiotic product LACTINA, a probiotic preparation of *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus helviticus*, *Lactobacillus lactis* *Streptococcus thermophilus* and *Enterococcus faecium*. Similar findings were also observed by Kunavue and Lien, (2012) in pigs.

We examined the energy profile and the mineral profile of the test group and the control group mice in order to analyze the effect of the test strain on the biochemical parameters of the host when it has to administered as a probiotic. Considerable difference in the lipid profile was observed between the test group and the control group whereas no significant change was observed in the protein level of the serum. The level of total serum triglycerides and cholesterol was significantly lowered in the test group which received the *Enterococcus faecium* MBTU-P1F1 as oral administration for 20 days. It was already learned from the *In vitro* studies that the test strain has the ability to deconjugate bile acids (due to the production of the BSH enzyme) and also to assimilate cholesterol from growth media. The lowering of the cholesterol level in the test group mice
can therefore be attributed to the bile salt hydrolase activity of the test strain. The result obtained in the in vitro study has thus been proved in the in vivo experiment. The result can be considered as a major finding of our study. We also observed higher HDL-cholesterol and a lower LDL-cholesterol level. Taken together the results, oral administration of the test strain, Enterococcus faecium MBTU-P1F1 can improve the lipid profile of the host animal by lowering the total cholesterol and total triglyceride level in serum. Similar results were observed by Zhu et al. (2009) in mice. They studied the cholesterol lowering properties of potential probiotic strains and found that Enterococcus faecium Y5 enriched yoghurt can significantly reduce the serum cholesterol level in mice. The energy profile study revealed that the test strain significantly improve the health status of the host animal with respect to the serum lipid level with no effect on the protein level.

The mineral profile of the test group mice and the control group showed an increase in the level of inorganic phosphorus and calcium in the serum. According to Capcarova et al. (2011) probiotic supplementation can help to digest and absorb more calcium and support bone metabolism resulting in higher bone density and strength in birds.

Immunomodulation in humans by orally administered probiotic microorganisms has received increasing interest recently. Various immune responses have been reported to be influenced by probiotics and these immunomodulatory effects have been proposed for several potential applications. The present study investigated the effects of oral supplementation of the potential probiotic Enterococcus faecium MBTU-P1F1 on the humoral and cell mediated immune response in mice.
Levels of circulating IgA, IgG, IgM and IgE give information on the effect of oral administration of *Enterococcus faecium* MBTU-P1F1 on systemic/humoral immune response. Remarkable increase in serum IgG and IgM were observed in the test group of mice when compared to control mice and the difference was statistically significant (P<0.05). It has been reported that production of large quantities of IgG in the serum are stimulated when food substances such as probiotics are absorbed into the blood and serum IgG antibody plays an essential role in the systemic immune response (Benyacoub *et al.*, 2005 and Sun *et al.*, 2010). The increase in the level of IgM and IgG can regarded as a positive influence of the test strain on the immune response of the host and would give protection against pathogenic microorganisms. According to Kimura *et al.* (2006) IgG, IgM and IgA are the main antibodies in the serum preventing the host from pathogenic microorganisms and are measured as markers of humoral immunomodulatory function. However only a slight increase in the level of IgA was observed in the present study. There are several studies revealing that oral administration of probiotics could trigger and stimulate the immune system underlying the intestinal mucosa thereby enhancing IgA production (Fang *et al.*, 2000). Secretory IgA (S-IgA) is the major class of antibodies present in the mucosal secretions of the gastrointestinal tract. IgA plays an important role in the first line immune defence protecting the mucosa against adherence and invasion by enteric pathogens. We examined the concentration of IgA in the intestinal fluid of the test mice and the control group to determine effect of the test strain on the local immune response of the host animal. Only a slight increase in the level of the immunoglobulin IgA in the intestinal fluid of the test group was observed in the study. Our study is in agreement with Sun *et al.* (2010) who observed no significant
increase in the level of IgA in the intestinal fluid of Balb/c mice fed with Enterococcus faecium (SF68). There are reports that Gram positive Lactic Acid Bacteria showed no immunostimulating effects on the secretory immune response in rodents and Scharek et al. (2005) also reported that Enterococcus faecium lacks mucosal immune stimulating properties as it is an autochthonous inhabitant of the mouse gut flora. There are evidences that S-IgA is not essential and that IgG alone can prevent mucosal infection. IgE is the antibody involved in Type 1 hypersensitivity. The serum concentrations of the immunoglobulin IgE was found to be more in the test group than in the control but statistical analysis revealed that the difference was not significant with that of the control indicating that the test strain will not trigger an allergic response in the host. Moreover we did not observe any adverse health reaction in the test mice group. Probiotic bacteria may affect host antibody responses to pathogens by causing increased production of total immunoglobulins or by selective enhancement of specific immunoglobulin isotype (Wagner et al., 2000).

Evaluation of Enterococcus faecium MBTU-P1F1 on the humoral immune response was further analyzed by examining the specific antibody response using SRBC as the antigen. Immunization with SRBC resulted in the appearance of anti-SRBC antibody in the serum which was indicated by the agglutination of SRBC by the serum collected from the animal groups. Significant increase in the antibody titre was observed for the test group. SRBC is a thymus dependent immunogen and hence the enhanced antibody response to the antigen can throw light to the overall improvement of the humoral acquired immunity by test strain (Yang et al., 2009).

Significant enhancement in the phagocytic activity of the test group was observed in the phagocytic assay with 33% enhancement in the
percentage of phagocytosis with respect to the control group. Result of NBT assay revealed the effect of the test strain in stimulating respiratory burst by the peritoneal macrophages. Phagocytosis is an early and crucial event in the host defence against invading pathogens. An enhancement in nonspecific immune phagocytic activity of granulocyte population in the blood of human volunteers after consumption of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* has been demonstrated (Schiffrin *et al.*, 1995). According to Kailaspathy and Chin, (2000) phagocytic activity is associated with natural immunity and phagocytes are involved in antibody immune responses as antigen presenting cells. We have demonstrated the ability of *Enterococcus faecium* MBTU-P1F1 in enhancing the phagocytic activity and the respiratory burst in Balb/c mice. The result gives information on the ability of the test strain to mediate cellular immunity and also in humoral immune response by serving as antigen presenting cells.

Delayed type hypersensitivity (DTH) response is a measure of the acquired cellular immunity and manifests T cell mediated immunologic memory (Yang *et al.*, 2009). To study the DTH response of the treated and control mice, SRBC was used to stimulate the T lymphocyte activity. Increased inflammation of foot pad was observed in the test group than the control group and the difference between the two groups was highly significant. Enhancement in the DTH reaction in mice due to probiotic consumption was demonstrated by Yang *et al.* (2009). The result obtained in the DTH response has once again proved the immunostimulatory function of the test strain.

Effect of probiotic supplementation on the lymphoproliferation of spleen has been examined by many researchers. Significant enhancement of the proliferative responses of spleen cells to concanavalin A (a T-cell mitogen)
and lipopolysaccharide (a B-cell mitogen) in mice given different probiotic strains were reported by Reid et al. (2003). It has been demonstrated by Yang et al. (2009) that Bifidobacterium adolescentis BBMN23 and Bifidobacterium longum BBMN68 can enhance proliferation lymphocyte in mice in the presence of concanavalin A. We examined the lymphoproliferative effect of the test strain Enterococcus faecium MBTU-P1F1 in Balb/c mice in response concanavalin A and lipopolysaccharide and the results were compared with the s lymphoproliferation of the control group. Even though there was increase in the lymphoproliferative response of the test mice than the control group, statistical analysis showed that the difference between the two groups were nonsignificant with P>0.05. Also the difference between the response to conconavalin A and lipopolysaccharide was nonsignificant. Kirjavainen et al. (1999) the effect orally administered viable probiotic and dairy lactobacilli on the mouse lymphocyte proliferation in response to different concentration of the mitogens conconavalin A and lipopolysaccharide. They found significant strain dependency in the ex vivo proliferation of mitogen stimulated lymphocytes. Also the proliferative effect was found to have affected by the concentration of the mitogens. We found only very low in the lymphocyte proliferation of the test group in response to the mitogens which could be attributed to the particular concentration of mitogens used in the study. Hence we cannot come to a conclusion regarding the effect of the test strain on splenic lymphocyte of the host animal.

The antioxidant property of liver tissue collected from the control group and the test group mice was examined. The test group which received Enterococcus faecium MBTU-P1F1 as probiotic showed more scavenging activity than the control group. The antioxidant property of the test strain was revealed in the in vitro studies and the finding has been further proved in
Balb/c mice. Lactic Acid Bacteria possess different antioxidative mechanisms such as reduced glutathione, superoxide dismutase, NADH oxidase, NADH peroxidise, thiol compounds, metal iron chelating ability, scavenge of reactive oxygen species and reducing activity (Meira et al., 2012). It has been proved in the present study that Enterococcus faecium MBTU P1F1 can enhance the antioxidant capability of liver tissue in Balb/c mice which in turn can improve the overall health status of the host preventing accumulation of toxic radicals in the body.

Conclusion

To conclude, our findings revealed many of health promoting attributes of the potential probiotic strain Enterococcus faecium MBTU-P1F1 characterized in the study. In vitro studies showed the ability of the strain to deconjugate bile salts through the production of bile salt hydrolase enzyme, its ability to assimilate cholesterol from growth media, production of β galactosidase enzyme, as well its ability to scavenge DPPH radical which indicates the production of antioxidants by the strain. In vitro studies further supported the cholesterol lowering property of the strain where we observed a more healthy lipid profile in the test group mice than in the control group. There was significant reduction in the serum cholesterol and triglyceride level. Effect of oral administration of the test strain on the immune response of the host animal showed the strain was capable of stimulating both the cell mediated and humoral immunity.

Further the examination of the antioxidant property was in support with the in vitro finding Enterococcus faecium MBTU-P1F1 produces antioxidants which can prevent accumulation of reactive oxygen species.